Quantification of a Potent 5-HT_{2a} Antagonist and an Active Metabolite in Rat Plasma and Brain Microdialysate by Liquid Chromatography–Tandem Mass Spectrometry

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A method based on liquid chromatography–tandem mass spectrometry and microbore column separation was developed for the quantification of a potent 5-HT_{2a} receptor antagonist (R)-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidine-methanol (I) and the desmethyl metabolite (II) in rat brain extracellular fluid (ECF) following microdialysis sampling. The analytical method was also applied to determining plasma concentrations of these compounds. The lower limit of quantification (LLQ) for each compound in microdialysis perfusate is 500 pg/mL, which translates to < 7 fmol (injected). The recovery of I and II for the microdialysis probe in brain ECF was 18.5 and 22.7%, respectively. The LLQ for each compound in plasma is 1 ng/mL. The inherent selectivity offered by tandem mass spectrometry eliminated chemical noise, thereby improving the detectability of these compounds. These methods were used to confirm that I and II penetrated the blood–brain barrier following administration of I to rats and enabled comparison of plasma and brain ECF concentrations. (J Am Soc Mass Spectrom 1997, 8, 371–379) © 1997 American Society for Mass Spectrometry

Microdialysis sampling of in vivo systems has emerged as a powerful tool for investigations of drug pharmacokinetics and metabolism [1–4]. The initial development of microdialysis sampling was for probing brain neurochemistry, usually for the measurement of endogenous neurotransmitters [5–10]. However, continued application of this technique has proven its use in sampling many other tissues and organs including skin [11], muscle [12–14], adipose [15–17], liver [13, 18–20] and biological fluids [18, 21–23]. Specific placement of the microdialysis probe within the organ or tissue accesses the extracellular fluid (ECF) for sampling small molecules, either endogenous compounds or drugs. Small molecules diffuse through the microdialysis membrane and are swept away by the perfusion medium, which ideally is prepared to match the ionic strength and pH of the extracellular space that is being sampled, thereby minimizing changes in the microenvironment adjacent to the microdialysis probe [1]. Although insertion of the probe is invasive and will alter the physiochemical environment in the immediate area of implantation, it does not impact the integrity of the whole animal.

Implantation of a microdialysis probe within tissue provides the capability of continuous sampling without the loss of fluids. This feature offers potentially greater temporal resolution than does serial sampling for monitoring drug concentrations, which is advantageous in pharmacokinetic and metabolism studies [3, 18, 22]. However, the inherent characteristics of microdialysis sampling present analytical challenges for measuring analyte concentration. Typically, to achieve the highest possible recovery of analytes from the matrix, the flow of the perfusion medium is 1 μL/min or less. To maintain the temporal resolution advantage requires the handling and subsequent analysis of a low volume sample that contains high salt concentrations and dilute analyte concentrations. Often, samples are analyzed by liquid chromatography (LC) with sensitive detection provided by electrochemical [5, 18, 24–27], ultraviolet [18, 28–30], fluorescence [31], or mass spectrometry [3, 4, 7, 22, 23, 32–34]. Capillary electrophoresis is well suited to the analysis of low sample volumes and is being increasingly utilized [7, 35].

As a potent and selective 5-HT_{2a} receptor antagonist, MDL 100,907 [(R)-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidine-methanol, (I)], shown in Figure 1, has been proposed, depending on the brain region, to either reduce or increase dopamine production [36]. A drug with this capability may be

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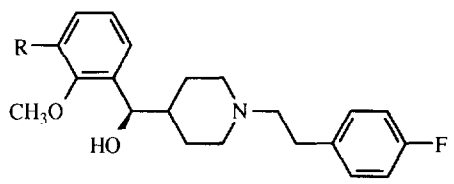

Compound	R	M.W.
I (*R*)	- OCH_3	373
II (*R*)	- OH	359
I.S. (*R,S*)	- H	343

Figure 1. Structures of compounds I, II, and I.S.

useful as a novel therapy for schizophrenic patients. Schizophrenia is unique in that as a disorder there are what are termed positive symptoms and negative disorders due to excessive and reduced dopamine activity within different regions of the brain [9]. Treatment for schizophrenia usually involves administering neuroleptics or typical antipsychotics, which are dopamine D_2 receptor antagonists. While attenuating the effects of the dopaminergic system, typical antipsychotics often exhibit negative side effects. These side effects include those manifested in the form of drug-induced Parkinsonism, which account for bradykinesia, tremor, and rigidity. Such side effects therefore, often limit the use of typical antipsychotics. Antagonists of 5-HT_{2a} receptors however, offer an alternative approach for reducing dopaminergic activity. Compound I exhibits 300–1000-fold selectivity for 5-HT_{2a} receptors (K_i = 0.36 nM) as compared to other neurotransmitter receptors (serotonin, 5-HT_{1c}; dopamine, D_2; adrenergic, α_1), and has been shown to increase dopamine efflux in the medial prefrontal cortex (mPFC) of the rat brain [36] and decrease the activity of ventral tegmental area dopamine neurons [37]. The ability to regulate activity selectively via 5-HT_{2a} antagonism is a strategy being pursued for the development of novel medications for the treatment of schizophrenia [9, 36].

Studies showing increased dopamine levels in the mPFC of the rat brain have previously been conducted after I was administered directly into the brain via the microdialysis probe [36]. In our studies, microdialysis sampling was used to obtain samples representative of the brain ECF following intravenous (i.v.) and oral administration of the drug to rats. A method based on liquid chromatography–tandem mass spectrometry (LC-MS/MS) was developed which enabled the quantification of I and II in both brain ECF (via microdialysis sampling) and plasma (samples serially collected). These experiments were performed to provide direct evidence as to the ability of I and II to penetrate the blood–brain barrier (BBB). Plasma samples were analyzed to compare the concentrations of I and II in the brain ECF. Although the sample processing for the two methods differed slightly, the selected reaction monitoring (SRM) detection was identical. This report describes the analytical methodology that was used to investigate the pharmacokinetics of I in the rat central nervous system (CNS) and plasma.

Experimental

Materials

Compounds I and II and the internal standard (I.S.) (refer to Figure 1) were obtained from Hoechst Marion Roussel Research Institute (Cincinnati, OH). Acetonitrile was high performance liquid chromatography (HPLC) grade (Burdick and Jackson, Muskegon, MI). Water was purified through a NANOpure II system (Barnstead, Dubuque, IA) prior to use. Artificial cerebral spinal fluid (aCSF) was used as the dialysis perfusate and consisted of 2.5-mM KCl, 1.18-mM $MgCl_2$, 1.26-mM $CaCl_2$, and 125-mM NaCl. All reagents used in the preparation of buffer solutions were of analytical reagent grade or better.

Instrumentation

Chromatographic separations were performed with a Michrom BioResources Ultrafast Microprotein Analyzer HPLC system (Michrom BioResources, Pleasanton, CA). Following chromatographic separation, 0.127-mm-i.d. Polyetherether ketone (PEEK) tubing delivered the effluent to the electrospray ionization source of a Finnigan MAT (San Jose, CA) TSQ 700 mass spectrometer via a Valco Instruments (Houston, TX) electric six port injector valve. This divert valve was installed to allow for automated control of the effluent flow to the mass spectrometer. The heated capillary was maintained at a temperature of 220 °C, and the nose cone needle was at 4.5 kV. Nitrogen was the sheath gas at a pressure of 38 lb/in.^{2}. Collision-induced dissociation (CID) of the protonated molecules of the analytes (m/z 374.1, 360.1) and the internal standard (m/z 344.1) caused fragmentation within the second quadrupole collision cell (Q2). Argon was the collision gas in Q2 at a pressure of 2.2 mtorr with a collision energy of 35 eV (E_{lab}). The product ion monitored by the third quadrupole (Q3) for both analytes and the internal standard was m/z 123 ($\pm$0.2 u; 0.5-s dwell time). The electron multiplier was set to 1600 V unless otherwise indicated. The samples were injected by a Gilson (Middleton, WI) model 231 autosampler.

Isocratic Chromatographic Conditions

The mobile phase consisted of acetate buffer–acetonitrile (42–58, v/v). The aqueous buffer was prepared by adding 10 mL of glacial acetic acid and 3.85 g of ammonium acetate to 990 mL of water. The HPLC column was an Alltima C8 (5 μm, 100 Å, 150 × 1 mm i.d.; Alltech, Deerfield, IL). The column was main-

tained at ambient temperature. The flow rate was 65 μL/min. All the chromatograms shown in the figures were generated without the use of a data smoothing routine.

Microdialysis

Animal preparation. Surgical implantation of the microdialysis probe (CMA/12, 2-mm probe length) consisting of a polycarbonate membrane (CMA/Microdialysis, Acton, MA) in the medial prefrontal cortex (mPFC) region of the rat brain was conducted as previously described [38]. After insertion of the microdialysis probe, the animal was placed in a plexiglass containment system (CMA/Microdialysis) and allowed to recover for 24 h prior to dosing with I.

Dose preparation and administration. For i.v. administration, animals (n = 3) were given a 5-mg/mL solution of I, which was prepared in a vehicle composed of 2% (w/w) citric acid, 0.6% (w/w) sodium citrate, and 0.5% (w/w) propylene glycol. A 50-mg/mL solution of I was prepared in 5.1% (w/w) citric acid, 1.6% (w/w) sodium citrate, and 41.4% (w/v) propylene glycol for oral administration in rats (n = 3). Fresh dosing solutions were prepared on each day of dosing.

Sample collection. The microdialysis perfusate was delivered via a syringe pump (CMA/Microdialysis) through the microdialysis probe at a rate of 1 μL/min. Dialysate samples were collected over 10-min intervals into 300-μL autosampler vials (Chromacol, Trumbull, CT) by using a CMA/170 refrigerated sample collector set at 5 °C. At the end of the study, dialysate samples were kept frozen at −20 °C until analysis. Blood samples (~ 100 μL/sample) were collected through the in-dwelling jugular vein cannula at 5 (i.v. dose only), 15, 30, 60, 90, 120, 180, 240, and 360 min postdose. Samples were collected into 64 × 10.25-mm heparinized Vacutainer® tubes (Becton Dickinson, Rutherford, NJ) and centrifuged at 3500 rpm (5 °C) for 15 min. The resulting plasma was pipetted into 1-mL microcentrifuge tubes and frozen at −20 °C until analysis.

In vivo recovery determination. Following insertion of the probe into the mPFC of the rat brain, the probe was perfused with aCSF at a flow rate of 1.0 μL/min. Prior to dosing with I, the in vivo delivery of the microdialysis probe was determined by perfusing the probe with a 50-ng/mL solution of I and II in aCSF. Delivery = $(C_{in} - C_{out})/C_{in}$, where C_{out} is the measured concentration of analyte in the perfusate coming out of the probe whereas C_{in} is the initial concentration of the analyte within the perfusate. Assuming that in vivo recovery is equal to delivery [39], the in vivo recovery of the probe was then calculated by using the formula $\%\text{recovery}_{\text{in vivo}}$ = delivery × 100.

Sample Preparation

Microdialysis perfusate samples. The only sample preparation required was the addition of 10 μL of I.S. solution to the collected perfusate sample (10 μL). The autosampler was programmed to draw 10 μL of I.S. solution (50 ng/mL) from a vial and introduce it into the vial containing 10 μL of perfusate. The subsequent sample was mixed with the syringe and a 10-μL aliquot was injected onto the chromatograph. Calibration standards used for the calibration curve were prepared in aCSF that was fortified with I and II. These standards were prepared and analyzed at concentrations of 0.5, 1, 5, 10, 25, 100, 250, 500, and 1000 ng/mL. Ten microliters of each calibration standard were placed in the autosample vials (in duplicate) for the LC/MS-MS analysis.

Rat plasma samples. To 25 μL of rat plasma was added 50 μL of acetonitrile fortified with I.S. at 50 ng/mL. Following the precipitation of plasma proteins, the sample was centrifuged for 10 min at 3500 rpm and the resulting supernatant was added to the autosample vial. The calibration standards were prepared as before after fortifying drug-free rat plasma (Pel Freez Biologicals, Rogers, AK) with I and II. Replicate calibration standards were analyzed at 1, 5, 10, 25, 100, 250, and 500 ng/mL. The injection volume onto the chromatographic column was 5 μL. The recovery of I, II, and I.S. from plasma following protein precipitation with acetonitrile was determined at 5, 50, and 500 ng/mL by comparing the peak areas of extracted plasma samples with the peak areas of identically treated fortified aqueous samples.

Assay Validation

The methods for analyzing both plasma samples and microdialysis perfusate samples were validated over the course of three days by the analysis of control samples at three concentration levels in the range of the calibration curve. Within-day reproducibility and accuracy was determined from the measurement of at least six replicates of quality control (QC) samples for perfusate samples and at least nine replicates for plasma samples. The between-day reproducibility was calculated by comparing three days of analyses run in replicates of greater than or equal to 6 on each day, for a total of at least 18 for the microdialysis perfusate samples and 24 for the plasma samples. The concentrations of QC samples prepared with the calibration standards and study samples were determined by using the logarithmic regression analysis equation.

Results and Discussion

Mass Spectrometry Analysis

To determine the mass spectrometric conditions that generated optimal detection of I and II, the analytes

were dissolved in mobile phase and continually infused into the ionization source of the mass spectrometer. Displayed in Figure 2 are the CID product ion mass spectra of the protonated molecules of I and II following the LC separation of 1 ng of each compound. For both analytes, the two major product ions were m/z 123 and 204. Proposed ion structures for the fragment ions of protonated compound I are depicted in Figure 2a. For those peaks labeled in each of the spectra with the same mass-to-charge ratio values, the structures of the ions are identical. The ion observed as a peak at m/z 232 for compound I shifts to an ion with m/z 218 for II. The fragmentation upon CID is centered around the basic nitrogen of the piperidine moiety. Whereas both compounds upon CID of $[M + H]^+$ yielded intense ion current at m/z 123, it was anticipated that the selectivity provided by SRM would minimize the required sample preparation and provide a low level of detection. Because both microdialysis perfusate and plasma samples were to be analyzed, an LC-MS/MS method was sought that would be identical for the analysis of compounds in each matrix. This would enable concurrent analysis of each matrix with changes required only for the autosampler procedures.

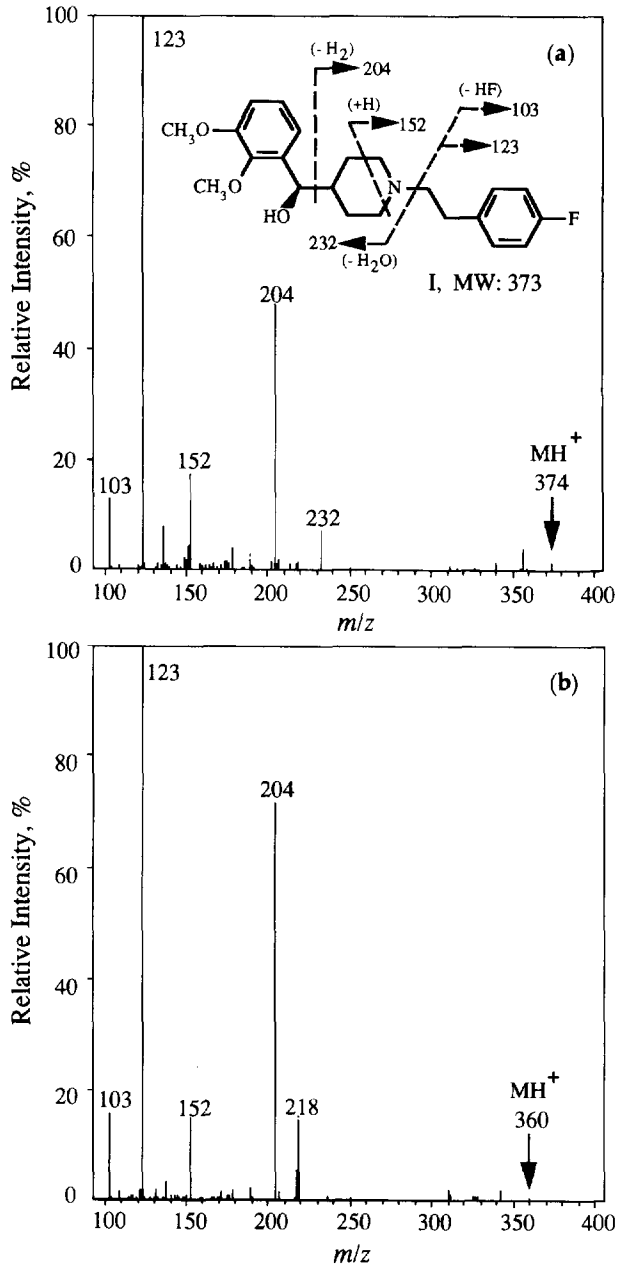

Figure 2. Product ion mass spectrum of the protonated molecules of (**a**) I and (**b**) II following CID with argon at a pressure of 2.2 mtorr and collision energy of 35 eV (lab).

Selected Reaction Monitoring versus Selected Ion Monitoring

The limit of detection (LOD) of an analytical method is indicated by the amount of material required to generate a chromatographic peak with an acceptable signal-to-noise (S/N). Two strategies can be invoked in an attempt to improve the LOD. First, one can try to improve the sensitivity of a method by selecting a detector that produces the largest response per amount of compound introduced. A second approach is to minimize the chemical noise, thereby improving the specificity of the analytical method [40]. SRM virtually eliminates chemical noise.

The suitability of this assay method for the analysis of perfusate samples is demonstrated in Figure 3, which shows the reconstructed ion chromatograms obtained from blank aCSF and aCSF fortified with I and II to a level of 1 ng/mL. The analysis of the 1-ng/mL sample corresponds to less than 14 fmol injected. A chromatographic peak with S/N ~ 4 is detected for both compounds. Displayed in Figure 4 are data obtained in the analysis of a 50-ng/mL aCSF sample following SRM and selected ion monitoring (SIM) of the protonated molecule. The absolute intensity of the signal in the SIM mode is more than 10 times that of SRM. Similar detection limits could be achieved for I by using SRM or SIM; however, high background ion current of unknown origin with m/z 360 throughout the chromatographic analysis precluded low level detection of II by

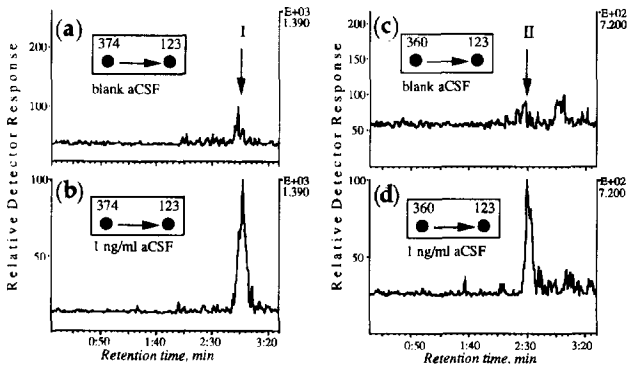

Figure 3. Chromatographic profiles obtained following the SRM analysis of blank aCSF (**a**) and (**c**) and aCSF fortified with I and II to 1 ng/mL (**b**) and (**d**). The arrows in panels a and c indicate where compounds I and II elute under these chromatographic conditions.

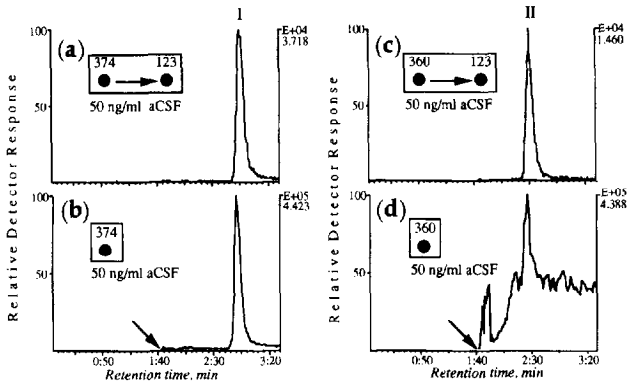

Figure 4. Chromatographic profiles obtained following the analysis of aCSF fortified with I and II. (**a**) and (**c**) were obtained following SRM analysis of aCSF fortified to 50 ng/mL, whereas (**b**) and (**d**) were obtained following SIM analysis of aCSF fortified to 50 ng/mL. The arrows in b and d indicate the time where the divert valve switched, directing effluent flow to the mass spectrometer.

SIM. This is evident from Figure 4c and d in which the ion chromatograms for SRM and SIM analysis of 50-ng/mL aCSF are displayed. Although similar S/N values are observed for I in the two mass spectrometry approaches (Figure 4a and b), clearly, SRM is superior for the analysis of II (Figure 4c and d). This background ion current of m/z 360 presumably is from a component found in the mobile phase as the ion current is quite constant. The chemical noise generated in the blank perfusate sample when analyzed by SRM is nearly identical for I and II (Figure 3a and c) because the additional selectivity offered by tandem mass spectrometry eliminates the high background ion current detected at m/z 360 in the SIM analysis.

It is of interest to deduce the source of the noise that is present in these analyses. The effect that increasing the electron multiplier voltage (EMV) has on the signal in either SIM or SRM mode helps define the noise as either chemical or electronic. Presumably, a majority of the background ion current generated in the SIM analysis is due to chemical noise, that is, noise resulting from the mobile phase. Ions are formed in the ion source and impact the conversion dynode of the electron multiplier. When the multiplier voltage is increased from 1000 to 1600 V, the background SIM ion current increases 75–100-fold (data not shown).

The source of background ion current in the SRM analysis of plasma extracts does not appear to be primarily from chemical noise. The reconstructed ion chromatogram (m/z 374 → 123) obtained in the SRM analysis of blank plasma extract with a multiplier setting of 1600 V is shown in Figure 5a. The chromatogram obtained under identical conditions for the 5-ng/mL plasma extract is shown in Figure 5b. This 5-ng/mL plasma extract was reanalyzed with the EMV set to 1000 V as seen in Figure 5c. There is only an approximate fourfold decrease in the signal of background ion current generated in the SRM analysis when the EMV is changed from 1600 to 1000 V as

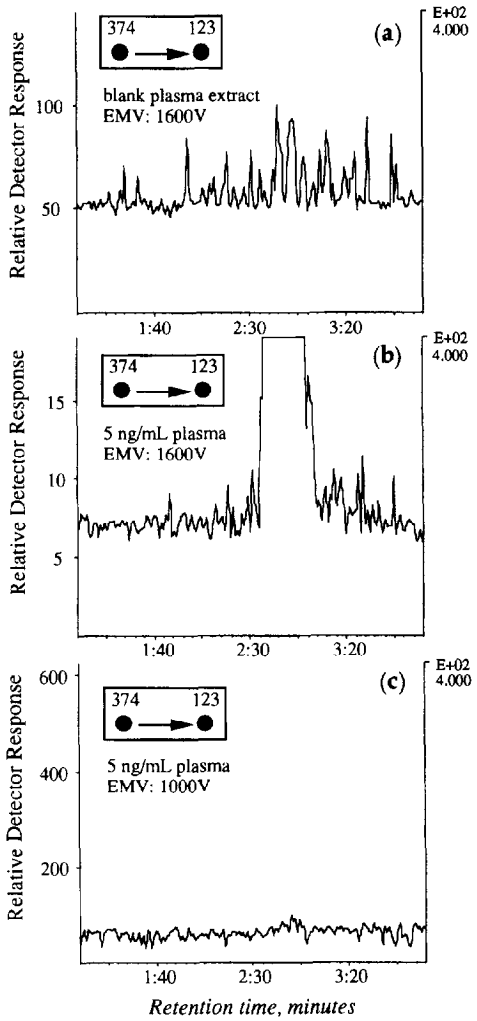

Figure 5. The SRM analysis of I in plasma extracts. (**a**) Blank extract, EMV at 1600 V; (**b**) 5-ng/mL extract, EMV at 1600 V; (**c**) 5-ng/mL extract, EMV at 1000 V. Note the absolute scale for the ordinate of each chromatogram is the same (4×10^2).

demonstrated in Figure 5. However, the analyte response (ion event at dynode) is not detected when the EMV is set to 1000 V in the analysis of the 5-ng/mL extract. These data indicate that nearly all of the noise in the background of the SRM analysis is electronic noise generated within the components of the circuit following the electron multiplier, and not chemical noise. Because the primary source of the noise is after the electron multiplier, a minimal increase in the background noise is observed when the EMV is increased, whereas there is a significant increase in detected signal when analyte ions strike the conversion dynode prior to generating the electron cascade.

Calibration Curves

Calibration curves for both I and II were constructed after analyzing standards in at least duplicate. Logarithmic regression analysis of the reconstructed ion chromatogram peak area ratios versus analyte concentration were performed. The correlation coefficients of

the calibration curves for I and II in aCSF were typically greater than 0.998. The correlation coefficients of the calibration curves for I in plasma were typically greater than 0.998, whereas for II in plasma, they were greater than 0.997.

Extraction Efficiency in Rat Plasma

For the analysis of I and II in rat plasma, the extraction efficiency (EE) of the protein precipitation with acetonitrile was determined at concentrations of 5, 50, and 500 ng/mL of each compound. Peak areas of triplicate extracted plasma standards were compared to peak areas of corresponding aqueous standards. The extraction efficiency for compound I averaged 79%, whereas the extraction efficiencies for II and the I.S. averaged 37 and 38%, respectively. Although all three compounds are structurally very similar, the recovery in plasma was significantly greater for I. We do not have an explanation for these observed differences in recovery. The data summarizing the extraction efficiency results are shown in Table 1.

Table 1. Extraction efficiency from rat plasma for I, II, and I.S. following protein precipitation with acetonitrile ($n = 3$)

Concentration of I and II (ng/mL)	Extraction efficiency (%)		
	I	II	I.S.[a]
5	69	35	35
50	87	41	38
500	81	34	41

[a]The internal standard concentration was 100 ng/mL for all concentrations of I and II.

In Vivo Recovery in Brain Extracellular Fluid

Calculation of the brain concentrations of both I and II were based on the in vivo recovery that was determined after implanting the probe into the brain. Based on the assumption that in vivo recovery is equal to in vivo delivery, which was determined in our experimental procedure [36], the recovery of I was 18.5%, whereas the recovery of II was 22.7%.

Accuracy, Precision, and Lower Limit of Quantification

The accuracy and precision of the method were determined from the analysis of three sets of QC samples for both aCSF samples and rat plasma samples. These data are summarized in Tables 2 and 3. Generally, the percent relative standard deviation (RSD) and percent relative error (RE) determinations for compounds I and II were similar for the analysis of aCSF. The lower

Table 2. Precision and accuracy data for determination of I and II in microdialysis perfusate

Concentration (ng/mL)	Within-day ($n \geq 6$)			Between-day ($n \geq 18$)		
	Found	RSD[a] (%)	RE[b] (%)	Found	RSD[a] (%)	RE[b] (%)
Compound I						
0.50	0.50	15.0	0.0	0.49	14.7	−2.0
25	24.6	6.7	−1.6	25.7	12.1	2.8
250	268.9	7.4	7.6	276.8	6.8	10.7
Compound II						
0.50	0.57	16.9	14.0	0.52	16.8	4.0
25	23.8	10.9	−4.8	24.1	14.5	−3.6
250	258.2	8.7	3.3	264.3	6.8	5.7

[a]Relative standard deviation.
[b]Relative error.

Table 3. Precision and accuracy data for determination of I and II in rat plasma

Concentration (ng/mL)	Within-day ($n \geq 9$)			Between-day ($n \geq 24$)		
	Found	RSD[a] (%)	RE[b] (%)	Found	RSD[a] (%)	RE[b] (%)
Compound I						
5	5.9	11.4	18.0	5.4	14.4	8.0
50	45.6	4.5	−8.8	51.0	11.6	2.0
500	541.5	2.5	8.3	532.0	5.5	6.4
Compound II						
5	5.3	19.0	6.0	5.9	17.6	18.0
50	47.9	19.5	−4.2	52.4	18.3	4.8
500	443.9	5.9	−11.2	507.6	12.2	1.5

[a]Relative standard deviation.
[b]Relative error.

limit of quantification (LLQ) for each compound in aCSF was 500 pg/mL. The precision in the measurement of II in plasma as indicated by RSD was slightly inferior to that of I in plasma. This is attributed to the lower EE of II from plasma following acetonitrile precipitation of plasma proteins. Variation in the recovery of II from sample to sample has a greater impact on RSD than it does in the case of I, which has a higher EE. Precision and accuracy data are shown in Table 3 for 5 ng/mL in rat plasma, although 1 ng/mL was readily detected and was used as the low level calibration standard. The RE and RSD for the 1-ng/mL plasma calibration standards were typically ~ 20% for each compound so 1 ng/mL was considered as the LLQ. The high organic content of the plasma extract limited our injection volume to 5 μL; larger injection volumes caused unacceptable broadening in the chromatographic peak shape.

The reproducibility of the absolute signal of I.S. measured as peak area was used to assess the precision of the microdialysis perfusate assay. This is useful in determining the effect that handling the small volume samples has on the measurement of I and II in perfusate. Generating a microdialysis sample from a rat involves collection of 10-μL perfusate over a 10-min period. The collection vials were capped and refrigerated to minimize sample evaporation prior to analysis. For the analysis, a 10-μL aliquot of I.S. solution was added by the autosampler to the sample and then mixed. Finally, 10 μL of sample was injected onto the chromatograph. Any variability that occurs within each of these steps along with variability in the mass spectrometer response will impact the signal generated for the I.S. Therefore, the precision determined for the measurement of the I.S. signal from study samples reflects the overall precision of the analytical procedure. Displayed in Figure 6 is a graphical representation of the precision of the experiment following the analysis of brain microdialysis perfusate samples collected from a rat that had been dosed with I. The I.S. peak areas for nearly all of the injections were within $\pm$10% of the mean. The decreases in the I.S. responses at injections numbers 19 and 20 may be due to a partially plugged capillary introducing the sample to the ionization source. The problem disappeared at injection 21. The concentrations measured at these two injections were considered valid because the drug levels determined were consistent with the trend evident in the brain ECF concentration–time profile. This suggests that the variability was in the mass spectrometer response and not due to sample processing. The use of the internal standard should compensate for this instrument variability, thereby minimizing the impact on the accuracy of the measurements. Typically, if a large response difference is noticed in a sample analysis, the data are evaluated to determine if reanalysis is necessary. Following the analysis of hundreds of samples via this method, we observed differences (> 20%) in the I.S. response (compared to the mean for the run) perhaps for 4% of the injections. Even with the large difference from the mean at injections 19 and 20, the relative standard deviation (%RSD) for the internal standard response detected in postdose study samples (n = 35) was 10.2%. The good precision determined for the I.S. response in the study samples suggests that little variability was introduced by the handling of the small volume samples. Therefore, in this study, the use of small volume samples did not negatively impact the precision of the measured analyte concentration. Similarly, the precision in the I.S. measurement from calibration curve or QC samples was determined. The RSD for these samples (n = 25) was found to be comparable at 12.3%. The mean value calculated for the internal standard signal in the postdose samples was 10.4% less than the mean value of the signal determined from calibration samples. These data indicate that the actual volume of microdialysate introduced into the vial either by the microdialysis syringe pump (postdose samples) or introduced manually for the QC samples (via a pipetter) differed slightly. No attempt was made to introduce a correction factor in the analysis of microdialysis study samples to compensate for apparent slight difference in sample volume relative to the volume of calibration standards.

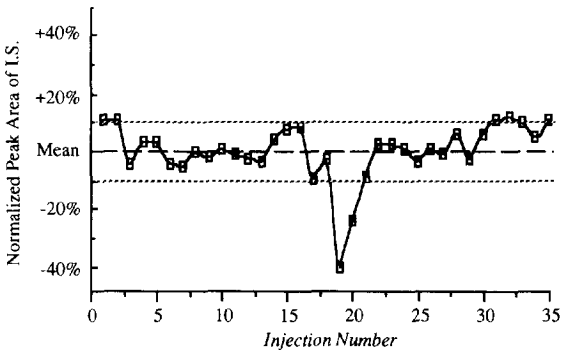

Figure 6. The normalized response of I.S. as indicated by chromatographic peak area, plotted as a function of injection number for the analysis of microdialysis perfusate collected for 10-min intervals following administration of I in rats. The overall %RSD was 10.2%. The dotted lines represent a deviation from the mean of 10%.

Application

Administration of I elicits CNS effects in vivo, indicating that the compound is able to penetrate the BBB. However, the extent to which I or an active metabolite II penetrates into the brain was unknown. Displayed in Figure 7 are the concentration–time profiles for I and II in both the brain ECF and plasma following a 5-mg/kg i.v. dose administered to a single rat. The brain ECF concentrations represent free drug, whereas the measurement of I and II in plasma represents the total drug concentration. Whereas the plasma protein binding of each compound is ~ 55% [38], comparison of the area under the curve (AUC) ratio

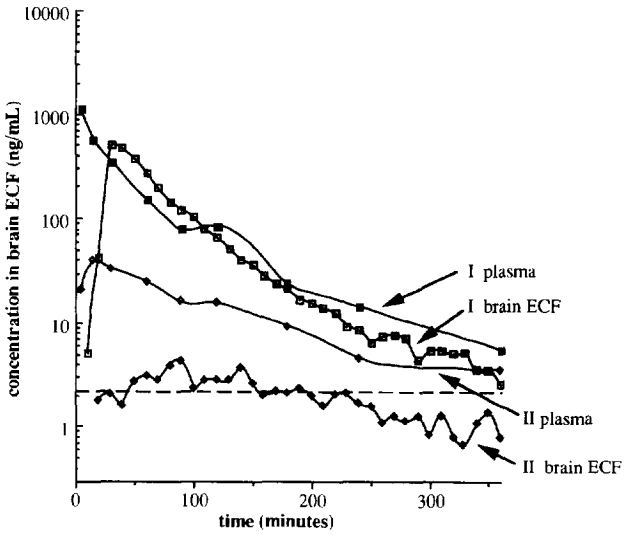

Figure 7. Semilogarithmic plot of plasma and brain ECF concentration time profiles for I and II following a 5-mg/kg i.v. dose administered to a rat. Note that the brain ECF concentrations represent the free concentration, whereas the plasma values represent the total drug concentration in plasma. The dashed line indicates the LLQ of I and II in brain ECF when taking into account the recovery of the microdialysis probe.

$AUC_{(brain)}/AUC_{(plasma)}$ for I and II will reflect on the relative ability of each compound to penetrate the BBB. The ratio of $AUC_{(I, brain)}/AUC_{(I, plasma)}$ in this study was 0.67, whereas this ratio for II was 0.16, indicating that I crosses the BBB more efficiently than II. The dashed line in Figure 7 indicates the LLQ of II in brain ECF after taking into account the recovery through the probe. Although there are some levels of II indicated in brain ECF which fall below the LLQ of the method, we show these concentrations in the time profile as the signal generated in the sample analysis was greater than that observed for blank perfusate sample. All of the concentrations depicted in Figure 7 for I in brain ECF and plasma, and for II in plasma are above the LLQ of the assay. A complete investigation into the CNS penetration of I and II following 5-mg/kg i.v. and 50-mg/kg oral dosing by using in vivo microdialysis sampling in rats (n = 3) is the subject of another report [38].

Conclusion

Methods based on microbore LC-MS/MS for quantification of compounds I and II in both microdialysis perfusate and rate plasma are described. The LC-MS/MS conditions were identical for each method. No sample processing was required in the analysis of aCSF samples generated in the rat brain microdialysis studies. Each sample was injected directly onto the LC column following the addition of the I.S. solution via the autosampler. An LLQ of 500 pg/mL was achieved for I and II in aCSF, which corresponds to 2.7 ng/mL (I) and 2.2 ng/mL (II) in brain ECF when taking into account recovery of the microdialysis probe. For the plasma analysis, the compounds were extracted by a simple acetonitrile protein precipitation procedure. Although the extraction efficiency of I was quite high in plasma, the recovery of II is poor. Nevertheless, data from the analysis of QC samples (both perfusate and plasma) are presented that demonstrate acceptable precision and accuracy for the method. The overall precision of the microdialysis perfusate sample analysis is determined by examining the %RSD of the internal standard response detected in study and calibration samples. Similar %RSDs and mean values in the I.S. detector response validate the sample handling procedures. These methods have been used to verify that I, and to a lesser extent its active metabolite II, penetrate the BBB following administration of I to rats. Understanding how brain and plasma levels correlate in rats may prove useful in predicting the extent to which the drug penetrates the human brain based on plasma concentrations.

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